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Elicitation of the immune response to *p*-phenylenediamine in allergic patients: the role of dose and exposure time

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Conflicts of interest
C.G., H.R., G.K., M.K., H.S. and G.F.G. are employees of the Procter & Gamble Company. The hair dye ingredient studied in this paper is currently used in commercial products marketed by the Procter & Gamble Company. P.-J.C. and B.B. participated as study directors and experts in skin sensitization. The authors alone are responsible for the content and writing of the paper.

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Summary

Background Usage of hair dye products containing p-phenylenediamine (PPD) is a concern for PPD-allergic individuals.

Objectives The present study investigates the role of dose and exposure time on elicitation of allergic contact dermatitis under conditions of permanent hair dyeing.

Methods Elicitation responses after application of a typical hair dye product containing 2% PPD for 30 min followed by rinsing were analysed in 38 PPD-allergic individuals with a documented history of hair dye-related allergy. Skin binding experiments *in vitro* were performed to distinguish the dose available for elicitation from the dose applied.

Results A positive reaction was elicited in 20 of 20 patients with grades ++ to +++ and 12 of 18 with grade + according to the classification of the International Contact Dermatitis Research Group. Under conditions of diagnostic patch testing (48 h exposure), the dose available for elicitation is more than 10-fold higher compared with the dose available for hair dyeing (30-min exposure, rinsing of product).

Conclusions This investigation demonstrates that under simulated hair dye use conditions the actual exposure to PPD is more than an order of magnitude lower than under diagnostic patch testing, although sufficient to elicit a clearly noticeable reaction in 84% of PPD patch test-positive individuals.

Mechanisms and factors influencing the elicitation response in sensitized individuals are not well understood. The response is time and dose dependent[1] and the threshold for elicitation decreases as the doses used to induce the allergy increase.[2] Consequently, clinical diagnosis aims at a high exposure scenario in order to yield maximal sensitivity for detecting all degrees of allergy in individuals seeking dermatological advice after having experienced skin problems. This is achieved by using the maximum nonirritant concentration of the suspected allergen under conditions promoting a high availability in the epidermis through occlusion and a relatively long exposure time. For that reason, an elicitation response is typically assessed after a single 48 h exposure application of the test item on the skin surface.[3–5] For p-phenylenediamine (PPD, an allergenic component in permanent hair dyes), the dose commonly applied for diagnostic purposes is approximately 400 μg cm^{-2} in white petrolatum when a Finn chamber is used[6] or 90 μg cm^{-2} in the 'True test' design with polyvidone as vehicle.[7,8] For these reasons the patch test represents the gold standard for the identification of human allergens and is the most relevant diagnostic tool to help a patient with contact dermatitis to avoid exposure to the causative agent.

Mechanistically, elicitation is affected by allergen-specific factors including the chemical potency, the type of exposure (e.g. time, intensity, frequency), anatomical region,[6] occlusion and vehicle.[9] This has been studied extensively for well-known contact allergens such as PPD.[6,10–16] Finally, the elicitation response is influenced by the strength of the individual's sensitization status, as it is mediated by the frequency and specificity of memory T cells.[1] This increases further the complexity of predicting under which conditions an elicitation response may occur.

For PPD, a common in-life exposure situation is permanent hair dyeing, because PPD is a frequently used hair dye precursor. Typically, the application to the hair is performed for a contact time of approximately 30 min (in the presence of hydrogen peroxide and other dye precursors under high pH conditions in a water-based formula), followed by rinsing

with water and shampoo.[17–19] As the described usage conditions are relatively uniform across all available products, hair dyeing with PPD can be considered a typical exposure scenario and a valuable model to investigate elicitation responses under real-life exposure conditions. Furthermore, PPD is generally regarded as the driving allergen in hair dye-related allergy and is considered sufficient to detect contact allergies to hair dyes.[20–22]

Consequently, the present work investigates how hair dye usage conditions (i) affect elicitation responses in allergic individuals with a documented history of hair dye-related allergy and different dermal response grades [+, ++, +++ according to the classification of the International Contact Dermatitis Research Group (ICDRG)] in a positive diagnostic patch test reaction to PPD, and (ii) compare with the diagnostic patch test conditions considering the dose/unit area relation between dose applied and dose available for elicitation on and in the skin by performing skin binding[15] (dermal absorption) experiments *in vitro*.

Materials and methods

The basic hair dye formula (without dye precursors and fragrance) used throughout the study reflects a typical basic formula of an oxidative hair colouring product (The Procter and Gamble Company, Wella Service GmbH, Darmstadt, Germany) and contained the following ingredients: aqua, cetearyl alcohol, sodium cocyl isethionate, sodium laureth sulphate, lanolin alcohol, ammonia, sodium sulphite, ascorbic acid, disodium ethylenediamine tetraacetic acid, benzoic acid, tocopherol. In order to obtain hair dye test product F, the dye precursors PPD, 2-methylresorcinol and 2-methyl-5-hydroxyethylaminophenol were added to the basic product at concentrations of 4%, 3·6% and 1·9%, respectively. The latter two hair dye precursors (couplers) were selected based on their negligible sensitization potency as determined in the local lymph node assay, each with an effective concentration (EC3) ≥ 50.[23,24] Immediately prior to application, hair dye test product F was mixed with the developer solution at a mixing ratio of 1 : 1 to yield the final on-head concentration of 2% PPD representing the maximally allowed concentration in the European Union. The developer solution (The Procter and Gamble Company, Wella Service GmbH) contained 6% hydrogen peroxide and the following ingredients: aqua, cetearyl alcohol, ceteareth-25, salicylic acid, phosphoric acid, disodium phosphate, etidronic acid. All other chemicals were of the highest grade available from commercial suppliers.

PPD free base (concentration 1%) in white petrolatum was purchased from Almirall Hermal GmbH (Trolab, Reinbek, Germany) and is referred to as patch test formulation H for the skin binding experiments.

Human elicitation study

The study was approved by the ethics committee of University Medical Center Groningen. Thirty-eight individuals were recruited (34 women and four men). They had been found to be allergic to PPD and had experienced an allergic reaction after use of hair dye products. The levels of response in a previous diagnostic patch test reaction (1% PPD in white petrolatum) were + (n = 18), ++ (n = 15) and +++ (n = 5) at day 3. A single dose of 100 or 150 mg cm^{-2} hair dye test product F containing 2% PPD was applied on their lower forearm with a van der Bend square patch test chamber (van der Bend, Brielle, the Netherlands) and fixed with Fixomull elastic tape (Beiersdorf, Hamburg, Germany). On the adjacent skin a similar patch test with the same basic formula, but without PPD and without couplers, was applied as a negative control. After 30 min (in one individual this was 5 min), the patch test chambers were detached and surface excess of hair dye test product F was removed from the skin surface by rinsing with water and shampoo. Reactions to hair dye test product F were recorded at day 2 and day 3 and graded according to the ICDRG criteria.

Skin binding (dermal absorption)

Experiments were conducted using flow-through diffusion cells following OECD guideline 428[25,26] and as described.[27–29] Briefly, pig skin samples (Schweizer Landedelschwein) were placed as a barrier between the two halves of the diffusion cell; the dermal side of the skin was exposed to receptor fluid representing the systemic compartment and the skin surface remained air exposed. PPD, spiked with 5 mCi [^{14}C]-PPD dihydrochloride (60 mCi $mmol^{-1}$; GE Healthcare UK Ltd, Little Chalfont, U.K.) was applied to the skin as described below. The receptor fluid was sampled at 16, 24, 40, 48, 64 and 72 h after application. The experiments were terminated after 72 h. All samples (such as skin surface excess, skin, and receptor fluid) were subjected to determination of radioactivity by scintillation counting. Detection limits were between 2·4 and 9·6 ng cm^{-2} for receptor fluid samples and between 3 and 10 ng cm^{-2} for the skin samples. Mass balance was calculated relative to the actual administered dose of [^{14}C]-PPD and only individual diffusion cells with a recovery of 100% ± 10% were considered valid.

Application of hair dye test product F: after mixing an equal amount of the hair dye formulation with developer, 150 mg cm^{-2} (corresponding to 3000 μg PPD cm^{-2}) of the mixture was spread evenly on the surface of the pig skin samples. The final formulation contained 2% PPD. After 5, 15, 30 or 60 min, the formulation was removed from the skin surface by washing in five steps with water and shampoo (The Procter and Gamble Company, Wella Service GmbH) and all samples were collected for analysis of radioactivity as described above.

Application of patch test formulation H: Finn chambers (0·7 cm^2 surface area) were filled with 20 mg of white petrolatum containing 1% PPD (corresponding to 40 mg formulation cm^{-2} and 400 μg PPD cm^{-2}) by weight, and subsequently fixed on the skin surface. After 48 h, Finn chambers were removed and formulation remaining on the skin surface was

Table 1 Elicitation responses of individuals with documented history of hair dye-related allergic contact dermatitis (n = 38) following occlusive exposure to 100 or 150 μg cm^{-2} hair dye test product F for up to 30 min

Number of subjects	Strength of previous diagnostic patch test response to PPD (at day 3)	Contact time (min) with hair dye test product F	Number of positively reacting/total subjects (at day 3)
5	+++	5–30	5/5
15	++	5–30	15/15
18	+	5–30	12[a]/18

[a]Six did not react after a contact time of 30 min; eight reacted with grade + and four with +/−. PPD, *p*-phenylenediamine.

removed with cotton tips. All samples were collected for analysis of radioactivity as described above.

Results

Elicitation responses in *p*-phenylenediamine-allergic individuals with a documented history of hair dye-related allergy under conditions similar to hair dye usage

The potential of a hair dye test product which contained 2% PPD (hair dye test product F) to elicit allergic contact dermatitis was assessed on the skin of 38 individuals who were diagnostic patch test positive to PPD and who had experienced hair dye dermatitis in the past. The strength of the previous diagnostic patch test reactions and results for product F at day 3 are summarized in Table 1. Of the 38 individuals tested, 32 reacted to hair dye test product F. All 20 individuals with a previous +++ or ++ patch test reaction to PPD showed a clear response but six of 18 individuals who had a + patch test reaction to PPD did not respond to product F within 30 min. A more detailed summary of the nonresponding individuals is given in Table 2. Two of these individuals were using hair dye products after their initial patch test and they appeared to be tolerant to a light shade. The other four avoided the use of hair dyeing products after their positive diagnostic patch test reaction to PPD. These results show that 84% of individuals showed positive elicitation upon a 30-min exposure with hair dye test product F, indicating a good correlation between the patch test results and the short-term exposure assay. No reactions were observed when the basic hair dye formula without dyes was applied.

Comparison of the *p*-phenylenediamine measured exposure level for hair dye usage and diagnostic patch test conditions

Skin binding studies were performed to compare the exposure scenario of diagnostic patch testing (48 h occlusive exposure to patch test formulation H with 400 μg cm^{-2} PPD in white petrolatum in a Finn chamber under occlusion) with that of hair dyeing (30 min open exposure to oxidative hair dye test product F with 3000 μg cm^{-2} PPD followed by rinsing with water and shampoo) (see Fig. 1). The mean ± SD amount of PPD associated with the skin (dermis and epidermis including the stratum corneum) was 109·6 ± 41·7 μg cm^{-2} for test patch formulation H and 5·9 ± 1·8 μg cm^{-2} for hair dye formulation F. In the receptor fluid (representing the systemic compartment), the mean ± SD PPD concentration was 95·5 ± 55·1 μg cm^{-2} and 0·9 ± 0·5 μg cm^{-2} for patch test formulation H and hair dye test product F, respectively. Accordingly, the measured exposure level (MEL) was calculated as the sum of the PPD concentration on/in skin and receptor fluid, i.e. 205·1 ± 46·6 μg cm^{-2} for the patch test formulation H and 6·8 ± 1·5 μg cm^{-2} for the hair dye test product F. Mean ± SD PPD concentrations in the surface excess (amounts recovered from the skin surface at the end of the exposure period), i.e. the amount of PPD not contributing to the MEL, were 206·3 ± 27·6 for patch test formulation H and 2662·6 ± 70·4 for the hair dye test product F, equivalent to 52% and 89% of the dose applied, respectively (Fig. 1).

Table 2 Summary of the six nonresponding individuals with a documented history of hair dye-related allergic contact dermatitis following occlusive exposure to hair dye test product F for up to 30 min

	Strength of response to product F (100 or 150 μg cm^{-2})		Strength of previous diagnostic patch test response to 1% PPD in petrolatum		
Individual	Day 2	Day 3	Day 2	Day 3	Comments
1	Negative	Negative	Negative	+	Tolerance to hair dye products[a]
2	Negative	Negative	Negative	+	Tolerance to hair dye products[a]
3	Negative	Negative	Negative	+	Avoided the use of hair dye products after the initial reaction
4	Negative	Negative	Negative	+	Avoided the use of hair dye products after the initial reaction
5	Negative	Negative	Negative	+	Avoided the use of hair dye products after the initial reaction
6	Negative	Negative	?+	+	Avoided the use of hair dye products after the initial reaction

[a]Tolerance to permanent hair dyeing with light shade. PPD, *p*-phenylenediamine.

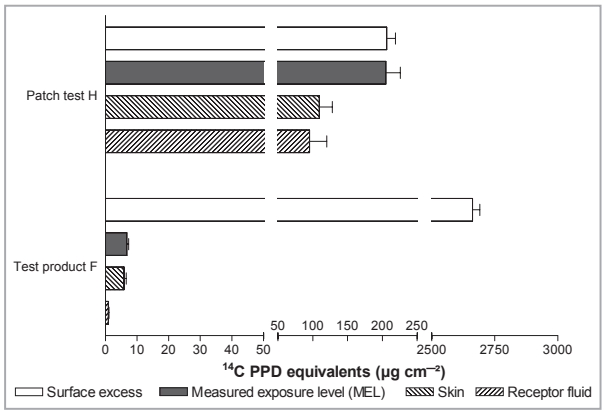

Fig 1. Comparison of exposure to *p*-phenylenediamine (PPD) under diagnostic patch test and hair dye use conditions. For patch test formulation H, white petrolatum containing 1% ^{14}C-PPD equivalent (400 μg cm^{-2} in Finn chamber) was applied to the skin surface for 48 h ($n = 5$) and subsequently the chamber and excess on the surface was removed. After mixing with hydrogen peroxide, hair dye test product F containing 2% ^{14}C-PPD equivalent (3000 μg cm^{-2}) was applied for 30 min and subsequently rinsed off with water and shampoo ($n = 6$). After 72 h, radioactivity was determined in the receptor fluid, skin and surface excess (white petrolatum removed from Finn chamber and skin surface for H or in rinsing solutions for F). The measured exposure level (MEL = sum of receptor fluid and skin) was calculated. Values represent arithmetic means ± SD.

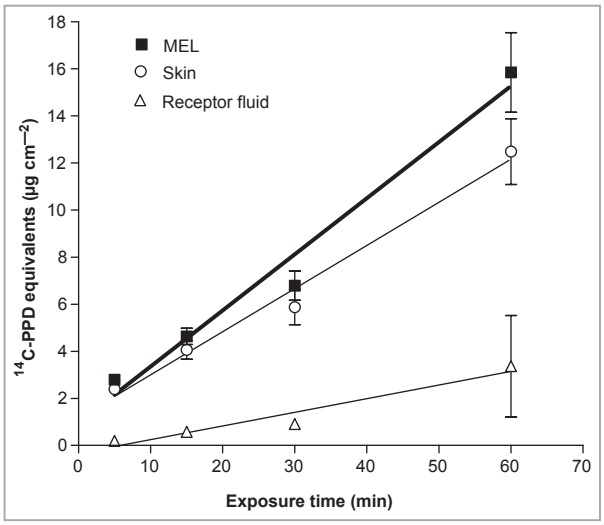

Fig 2. Time-dependent increase of *p*-phenylenediamine (PPD) exposure in skin and system. After mixing with hydrogen peroxide, hair dye test product F containing 2% ^{14}C-PPD equivalent (3000 μg cm^{-2}) was applied on the surface of skin samples for 5 ($n = 4$), 15 ($n = 6$), 30 ($n = 6$) and 60 ($n = 5$) min, and was subsequently rinsed off with water and shampoo. After 72 h, radioactivity was determined in receptor fluid and skin. The measured exposure level (MEL = sum of receptor fluid and skin) was calculated. Values represent arithmetic means ± SD.

Exposure time-dependent increase of the measured exposure level for *p*-phenylenediamine

MELs for PPD were determined following application of the hair dye test product F for increasing exposure durations of 5, 15, 30 and 60 min (Fig. 2). An exposure time-dependent increase of the MEL was observed with a correlation coefficient (r^2) of 0·98. Concentrations of PPD detected in the receptor fluid contributed only to a lesser degree to the MEL (with 7% at 5 min up to 21% at 60 min) than the corresponding concentrations on/in the skin. PPD concentrations in both compartments correlated well with the exposure time ($r^2 = 0{\cdot}94$ for the receptor fluid and $r^2 = 0{\cdot}99$ for the skin).

Discussion

In this paper, the relationship between positive elicitation responses with the contact allergen PPD both in diagnostic patch testing and in the (simulated) real-life situation of permanent hair dyeing was investigated. For that purpose, 38 individuals were selected with a PPD-related contact allergy corresponding to a history of hair dye product usage and a documented analysis of their patch test response upon diagnosis.

Firstly, it was asked if all 38 individuals would develop an elicitation response upon exposure to a hair dye product applied for 30 min (similar to real-life conditions) with a maximum realistic PPD concentration of 2%. All individuals who had a diagnostic patch test reaction with grade +++ or ++ developed an elicitation response at day 2 and day 3 (Table 1). Similar observations were made by Jowsey *et al.*[14] Although they applied a hair dye product containing a four-fold lower PPD dose (0·5%) compared with our study, they found that more than 50% of PPD-allergic individuals with a ++ reaction in the original patch test and more than 90% with +++ reactions reacted after 30 min.

In the present study, 12 of 18 individuals (67%) who had a diagnostic patch test grade + hair dye-related PPD allergy developed an elicitation response to the 30-min exposure to oxidative hair dye test product F at day 3 (Table 1), as expected considering their disease history with relevant symptoms in relation to hair dyeing. These data confirm that application of a hair dye product containing 2% PPD elicits an immune response in 84% of PPD diagnostic patch test-positive individuals.

For the six nonreacting individuals (16%), further analysis of their disease history revealed that two were still dyeing their hair (Table 2), indicating that the previous + patch test result was of no current relevance for hair dyeing as they did not react to a PPD concentration of 2% under simulated hair dye use conditions. This finding is also supported by Jowsey *et al.*[14] who found that none of the PPD-allergic individuals with a + diagnostic patch test response developed an elicitation reaction following a 30-min exposure to the hair dye product containing 0·5% PPD and only two of 15 reacted to a product with an unknown higher PPD concentration. In a study with 33 PPD-allergic patients, patch test results were of current relevance for 20 of 33 patients experiencing hair dye

dermatitis at the time of the patch test.[30] In line with our results, there was no current relevance of the patch test results for five of 33 patients as two were presently using PPD-containing hair dyes without any symptoms and three had previously dyed their hair with PPD-containing hair dyes without experiencing hair dye-related contact dermatitis.[30] Furthermore, a retrospective analysis in dermatology patients with a PPD-related allergy revealed that 73% of the + responders to the diagnostic patch test were still dyeing their hair while only 49% of the ++ responders and none of the +++ responders did so.[13] Furthermore, the elicitation threshold dose was found by Sosted *et al.*[6] to vary among PPD-allergic individuals: under diagnostic patch test conditions only a small number of patients (one of 15) reacted to a very low PPD dose of 0·0038% while with increasing PPD doses up to 0·5% the majority (87%) showed positive elicitation reactions.

Secondly, we investigated how hair dye use conditions compare with the conditions of diagnostic patch testing. We were interested in the differences between the dose applied and the dose actually available on and in the skin for elicitation. Therefore we used skin binding (dermal absorption) studies to correlate the positive elicitation reactions in PPD-allergic individuals to the actual MEL after removal of the surface excess instead of correlation to the dose applied.

We found that the MELs under hair dyeing conditions were more than an order of magnitude different from those under patch test conditions (6·8 μg cm^{-2} vs. 205 μg cm^{-2}, respectively, see Fig. 1). The MEL here is in same order of magnitude as PPD MELs calculated from published data on dermal penetration obtained under hair dyeing conditions, i.e. 16·1 and 21·9 μg cm^{-2} (see Table 3) for human skin and pig skin, respectively.[18]

As the applied concentrations under both scenarios of the current study were relatively high (3000 μg cm^{-2} for hair dyeing conditions and 400 μg cm^{-2} for diagnostic patch testing) and not likely to limit the maximum potential absorption, we considered the impact of the exposure time as a key factor for the observed differences in the MEL. A close correlation between exposure time and the number of positive reactions in PPD-allergic individuals is reported for PPD under hair dyeing conditions[14] as well as under patch test conditions.[11,12] In line with these findings, the current skin binding studies demonstrated a linear correlation between the exposure time and the MEL obtained experimentally under hair dyeing conditions (Fig. 2), i.e. application of the same dose for increasing contact times led to corresponding increases of the MEL.

Differences in skin metabolism of PPD were considered unlikely as no phase I skin metabolism has yet been reported and phase II skin metabolism (i.e. N-acetylation) of aromatic amines including PPD is well described in general and under hair dyeing conditions.[31–34] Correspondingly, N-acetylation is also very likely to occur under diagnostic patch test conditions.

Skin binding experiments in rats have recently been used to compare PPD concentrations retained in the skin after single or repeated short-term exposures to a hair dye formulation.[15] After a single application of 0·35% PPD for 5 min under conditions slightly deviating from product usage (skin rinsing with detergent prior to application, occlusion for 24 h, rinsing with water only after application), a MEL of 5·3 μg cm^{-2} (5·19 μg cm^{-2} absorbed plus 0·14 μg cm^{-2} in stratum corneum after the same experimental period of 72 h) was derived. In the present study, the MEL after 5 min exposure to hair dye test product F was 2·3 μg cm^{-2} (rinsing with water and shampoo, no occlusion, pig skin; see Fig. 2) and thus is very close to the published findings. When the frequency was increased to three daily exposures the MEL increased correspondingly to 14·8 μg cm^{-2}.[15] However, the relevance of daily exposures to PPD is unclear, as permanent hair dyes have a frequency of use of once every 4–6 weeks. Chemicals remaining on and in the stratum corneum and epidermis will be removed by continuous outward proliferation, differentiation and desquamation processes within a period of approximately 2 weeks for the stratum corneum alone and 4 weeks including the entire epidermis.[35]

So far, we found that exposure dose and time have a major impact on the MEL and thus on the elicitation response together with the degree of sensitization (as assessed by the diagnostic patch test response). In Table 3, the MEL for diagnostic patch testing and hair dyeing determined in our study was further compared with published data as well as with

Table 3 Overview of exposure conditions for hair dye products and diagnostic patch test

	Present data: hair dye test product F	Hueber-Becker *et al.*[18]	Krasteva *et al.*[36]	Present data: patch test formulation H
PPD concentration, %	2	2	0·1	1
Dose applied, μg cm^{-2}	3000	400	45·7	400
Exposure time, h	0·5 (rinsing)	0·5 (rinsing)	48	48
Measured exposure level, μg cm^{-2}	6·8	16·1 and 21·9	23·4[a]	205·1
Application conditions for elicitation testing	Occluded for 0·5 h (rinsing)	ND	Nonoccluded	Occluded for 48 h
Number of reacting/total subjects	32/38	ND	27/34	NA
Cumulative percentage of subjects reacting	84	ND	79[b]	100[c]

[a]Value for open application was estimated by using a factor of 1·95 between applied dose/measured exposure level for 48 h exposure under occlusion derived from patch test formulation H (400/205·1 μg cm^{-2}). [b]At an applied PPD concentration of 1·5% 34 of 34 subjects reacted. [c]Based on history of patch testing. PPD, *p*-phenylenediamine; NA, not applicable; ND, not done.

estimated MEL data from 48 h exposure to hair dyes. As the MEL is approximately 50% of the dose applied under diagnostic patch test conditions, this relation was also assumed for a 48 h exposure to PPD in a hair dye product applied nonocclusively, representing a conservative approach (Table 3). This indicates that the MEL of 23·4 μg cm^{-2} from an applied dose of 45·7 μg cm^{-2} or 0·1% PPD for 48 h can be considered as being in the same order of magnitude as the MELs of 16·1, 21·9 and 6·8 μg cm^{-2} from applied doses of 400 and 3000 μg cm^{-2} for 30 min (Table 3). Under both conditions, the elicitation response of the PPD-allergic individuals with a history of hair dye contact dermatitis was about 80%, with 32 of 38 in our study and 27 of 34 in the study of Krasteva *et al.*[36] The diagnostic patch test response in that study was: eight +++, 24 ++ and two + (see Table 3).

In summary, the data indicate that under simulated hair dye in-use conditions (including a 30-min application time) the actual exposure to PPD is more than an order of magnitude lower than under diagnostic patch testing, although sufficient to elicit a clearly noticeable reaction in 84% of PPD patch test-positive individuals.

What's already known about this topic?

- Usage of hair dye products containing *p*-phenylenediamine (PPD) is a concern for PPD-allergic individuals.

What does this study add?

- This study found that under in-use conditions the actual exposure to PPD is more than an order of magnitude lower than under diagnostic patch testing, although sufficient to elicit a clearly noticeable reaction in individuals with a moderate and strong allergy against PPD based on diagnostic patch test grades.

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